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The Relation of the Membrane Potential of Nitella to Photosynthetic Processes and External Conditions

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THE RELATION OF THE MEMBRANE POTENTIAL OF NITELLA TO PHOTOSYNTHETIC PROCESSES AND EXTERNAL CONDITIONS

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TABLE OF CONTENTS

INTRODUCTION

I. REVIEW OF LITERATURE

A. Origin of the resting membrane potential

One of the features of the intact plasmalemma is the presence of an electric potential difference across its thickness, with the inside of the membrane negative and the outside positive. The maintenance of this electrical gradient was at first thought to be the result of the passive diffusion of the major ions through the membrane. An equation was thus derived by Goldman (1943):

$$E_m = \frac{R\,T}{F} \ln \frac{(P_K K_o + P_{Na} Na_o + P_{Cl} Cl_i)}{(P_K K_i + P_{Na} Na_i + P_{Cl} Cl_o)}$$

where R = universal gas constant, 1.99 cal/mole degree
T = absolute temperature, ^oK
F = Faraday constant, 23,060 cal/mole volt
P = permeability of the membrane to the ion, cm/sec
K_i, K_o, etc. = concentration of the ion inside and outside the cell, respectively, molar
E_m = resting potential, volts.

However, through the study of the ionic composition of cells and the measurement of electric potentials, it was found that passive diffusion was generally not the principal mechanism accounting for the potential. There is a variety of evidence for active transport processes in organisms. If the charges transferred by this active transport process represent net charge transfer then the mechanism is called

an electrogenic pump.

An adequate description of any electrogenic mechanism requires the gathering of three types of information: the first one is the identification of the ions transferred by the electrogenic pump(s); the second is the identification of the substances providing the energy for the pump(s); and the last one is the chemical or physical nature of the mechanism by which the energy source is used to drive the active transport.

In animal cells the energy for all ion transport is believed to be ATP, or at least linked to ATP to a certain degree (reviewed by MacRobbie, 1970). Through the utilization of ATP a high concentration ratio of K^+/Na^+ is maintained in the cell, with Na^+ being constantly pumped out of the cell, and K^+ being pumped in at a slower rate; because of the imbalance of charge the pump is electrogenic and thus an electric potential is established across the membrane. In plant cells, of which most of the information has been obtained from characean species and Hydrodictyon africanum, a high concentration ratio of K^+/Na^+ is also observed and the K^+ influx and Na^+ efflux are also believed to be ATP dependent (MacRobbie, 1970, 1971). But the problem seems to be more complicated, since Na^+ extrusion is very small in Nitella (Barr, 1965). It seems unlikely that the Na^+ extrusion pump could be the principal electrogenic mechanism. Kitasato (1968) concluded that the active H^+ extrusion might be the mechanism responsible for the maintance of the electric potential.

His conclusion was based on the fact that the membrane potential is largely pH dependent in the range 4-6, and the total conductances of K, Na and Cl are negligible compared to the measured plasmalemma conductance. He therefore proposed a H^+ extrusion pump balanced almost entirely by the passive H^+ influx. The detection of acid and alkaline zones along the length of Nitella cells with the pH indicator dye, phenol red, (Spear, Barr and Barr, 1969) supported the existence of an H^+ extrusion pump.

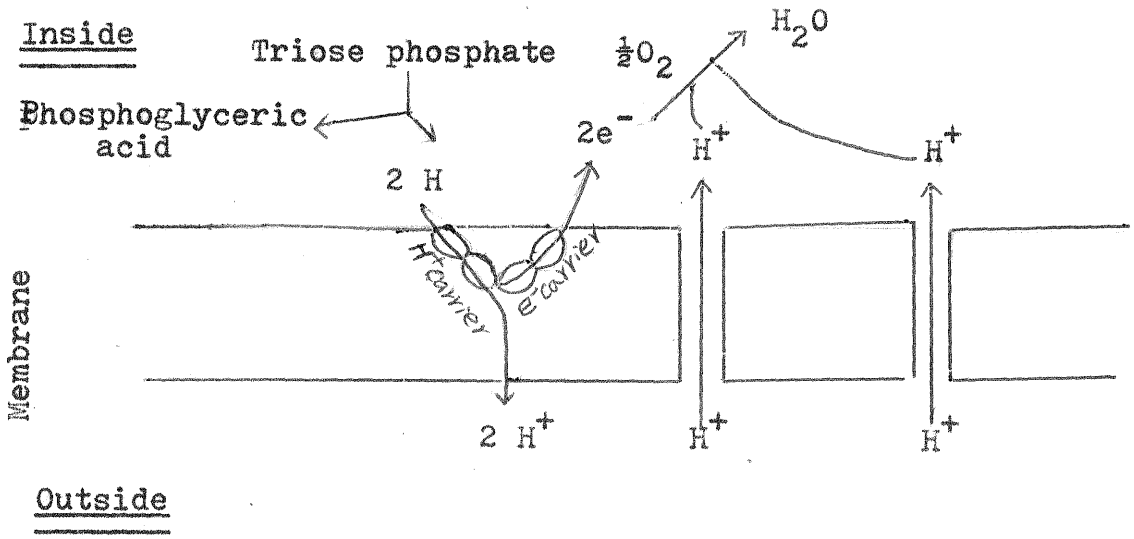
B. The role of the H^+ extrusion pump in controlling internal pH

Besides establishing an electric potential across the plasmalemma, as the Na^+/K^+ pump does in the animal cell, H^+ extrusion pump may also be concerned with the internal pH of the cell. Since the level of the resting membrane potential in Nitella is quite variable among cells and often varies with time in a given cell, the idea that the primary role of the H^+ extrusion pump is to control the internal pH of the cell seems to be correct (Raven and Smith, 1973). Resting membrane potentials usually range between -100 and -200 mV in internodal cells of characean species. It should be noted that the absolute rate of H^+ extrusion must correspond to the magnitude of passive H^+ influx if the internal pH is to be kept relatively constant. Therefore at high external pH there might be very little H^+ influx so that even a slowly working H^+ extrusion pump would be able to control the pH

and also sustain the negative potential.

C. Possible mechanisms of H^+ extrusion

The energy source for the H^+ extrusion pump has so far remained unknown. However, Spear et al (1969) have suggested a mechanism by which the photosynthetically-produced triose phosphate would serve as an oxidizable substance leading to the separation of H^+ and e^- in the membrane without phosphorylation taking place (see diagram):



According to this mechanism, triose phosphate leaks from the chloroplasts to be oxidized in the cytoplasm by NAD with the generation of $NADH_2$ and phosphoglycerate. Phosphoglycerate in turn diffuses back into chloroplasts to be reduced by the photochemically generated $NADPH_2$. The indirect evidences which support this mechanism are that triose phosphate is well known to be formed rapidly in the chloroplasts, and it can

pass rapidly through the chloroplasts envelope (Robinson and Stocking, 1968; Heber and Santarius, 1965; Urbach et al., 1965). Triose phosphate dehydrogenase (Benedetti and Emmelot, 1968) and NADH-cytochrome b_5 reductase, as well as cytochrome b_5 (Emmelot et al., 1964b) have been detected in the plasmalemma of liver cells. Although there is no evidence as yet that this system exists in the Nitella plasmalemma, at least these observations suggest the possibility of it.

According to Mitchell's chemi-osmotic hypothesis (Mitchell 1968) phosphorylation taking place in the inner membrane of chloroplasts and mitochondria is driven by the pH gradient across the membrane. Conversely ATP hydrolysis could be used to separate H^+ and OH^- across the plasmalemma. This suggests that ATP may be a possible energy source for the H^+ extrusion pump in Nitella. Lack of definite evidence prevents one from choosing between the redox mechanism described above and the ATPase mechanism. There is no direct evidence for either mechanism in Nitella.

D. Factors affecting the level of the resting membrane potential:

Many environmental factors have been investigated for their effects on the resting membrane potential. The effects of light, external pH, HCO_3^-, and CO_2 on the resting membrane potential are to be reviewed here with an emphasis on the idea that all these effects might be explained on the basis of how they might affect the internal pH, which in turn would

influence the rate of H^+ extrusion. The other factor to be considered is how fast H^+ enters the cell, with the stabilization of the resting potential being established when these inward and outward H^+ fluxes become balanced.

1. Light

Light-induced changes in the membrane potential of plant cells, most often transient, are very confusing since both hyperpolarization and depolarization are observed depending on the specific conditions (compiled by Rent, 1970). Nishizaki (1968) observed that DCMU (3-(3,4-dichlorophenyl)-1,1dimethyl urea) reversibly abolished both the oxygen evolution and the light-induced changes of the membrane potential level in Chara; also an enhancement of the light-induced response of the potential was observed when bicarbonate ions were present. He therefore suggested that some photosynthetically-produced carbon compounds were responsible for the light-induced changes. His conclusion was consistent with the H^+ extrusion mechanism suggested by Spear et al. (1969). Spanswick (1972) apparently has eliminated the possibility that a decrease in the K^+ concentration at the outer surface of the plasmalemma brought about by light-dependent K^+ influx could be the cause of light-induced hyperpolarization. The light-induced increase of the permeability coefficient of Na^+ observed by Hogg et al. (1969) cannot account for the resting membrane potential changes,

since Na conductance is very small in the Nitella plasmalemma (Kitasato, 1968). As to the effect of light on the membrane potential in relation to the internal pH, it is known that chloroplasts take up H^+ when illuminated (Jagendorf and Uribe, 1967). Therefore, in light one would expect the internal pH of the cell to be higher than the pH optimum for the operation of the H^+ extrusion pump. The pump will then be slowed down and a depolarization would be predicted. On the other hand, if the pH is initally low, the pH rise brought about by photosynthesis could result in a more favorable pH for the operation of the H^+ extrusion pump and a hyperpolarization would be expected. This suggestion implies that the H^+ extrusion pump operates maximally, not when the internal pH is lowest, but at some intermediate pH. This idea is offered because it is known that characean species do not grow in acidic habitats and may not be capable of surviving when the internal pH is artifically lowered.

It has been shown in isolated chloroplasts of Acetabularia that the ambient pH exerts a significant effect on the rate and distribution of carbon dioxide incorporated into the various photosynthetic intermediates (Dodd and Bidwell, 1971). In intact cells, the cytoplasmic pH as affected by both the light-induced H^+ uptake by chloroplasts and the rate of H^+ extrusion by the cell membrane would thus influence the destiny of

CO_2 being fixed. It is also possible that the rate and pattern of photosynthetic carbon compound formation will in turn influence the internal pH of the cell. The concept of Raven and Smith (1973) that the primary role of the H^+ extrusion pump is to control the cytoplasmic pH implies that the H^+ pump is not dependent upon specific carbon compounds per se but is dependent on any pH changes which occur as a consequence of photosynthesis including the nature of the carbon compounds formed. Such a conclusion also suggests that a given aspect of photosynthesis (e.g. carbon fixation, photophosphorylation, etc.) may alter the internal pH to a level which is not necessarily consistent with the maximal rate of that process. If these interwoven relationships between the internal pH and the carbon distribution pattern could be analyzed, the conflicting results obtained by changing the illumination or the carbon supply might be more understandable.

2. External pH

Kitasato (1968) has shown that the resting membrane potential is highly sensitive to external pH changes in the range 4 to 6. The decrease in pH brings about a substantial depolarization. He explained this phenomenon by reasoning that at low external pH the passive H^+ influx is too great to be adequately balanced by the H^+ extrusion

pump. For the depolarized membrane the electrical driving force for passive H^+ influx is less and flux balance is possible. If Kitasato's argument is correct, one would expect to see a large increase in the membrane resistance when the pH is increased from 5 to 7 in the external solution since H^+ is thought of as the main current carrier. However, this is true only in dark but not in light (Spanswick, 1972). The membrane resistance is low and insensitive to the external pH changes in light. These observations led him to suggest that the low value of the membrane resistance in light is due to something other than the passive movement of H^+ ions inwardly. This means that measured membrane resistances cannot be used to evaluate the passive properties of the membrane with any degree of confidence. Passive H^+ influxes calculated from pH gradients and membrane resistances are therefore open to serious question. There is evidence, however, that a substantial H^+ influx does occur when the external pH has been lowered to 4.7 (Rent et al., 1972), as determined by the rate of pH increases of the external solution. On this basis, one can assume that for external pH values below 5 there is a net H^+ influx and the internal pH will be lowered. Technical difficulties have prevented the actual measurement of cytoplasmic pH changes in Nitella. The effects of raising the external pH to alkaline values is discussed in the next section.

3. Bicarbonate ions

Some studies have brought attention to the possible role of bicarbonate in the electrogenic process. The possibility of the active inward transfer of HCO_3^- has been proposed by Hope (1965). He found that the membrane potential of Chara was hyperpolarized by 60mV when Cl^- in external solution was partially replaced by HCO_3^-. He therefore proposed an electrogenic active system which pumps bicarbonate into the cell. This point of view was supported by the finding of Raven (1967) in which he showed Hydrodictyon africanum could photosynthetically fix HCO_3^- when it became the only carbon source available. However, the carbon fixation rate was much slower with HCO_3^- than with CO_2 at the same concentration (Raven, 1967). On the basis of kinetic evidence, Raven suggested there is a light-dependent step in the HCO_3^- uptake which is in addition to that required for CO_2 assimilation. Although he agreed that there might be an electrogenic HCO_3^- pump, Raven pointed out that the duration of the hyperpolarization caused by HCO_3^- is very short (1-2hours) but the HCO_3^- entry is invariant with time for at least 10 hours. Smith (1968) indicated that the degree of hyperpolarization in Chara australis and Nitella translucens appears to be very similar, even though Chara cells absorb bicarbonate five times faster than do Nitella cells. This suggested that the hyperpolarization caused by HCO_3^- and the entrance of HCO_3^-

into the cell might be two completely separate phenomena.

It should be noted that in the work done by Hope (1965) and Raven (1967) significant pH increases (one unit or more) were always concomitant with the HCO_3^- addition. Spanswick (1970) found that a hyperpolarization can also be produced in Nitella by raising the pH to 7 or 8 through the addition of buffers other than bicarbonate. Bicarbonate ions have no effect when the cells have previously been in a solution buffered at the same pH (7.2) by another substance. If indeed HCO_3^- acts as a buffer in hyperpolarizing the resting membrane potential, its influences on the external pH and the internal pH can be explained: When the pH of the external solution is high, the passive H^+ influx in the externally visible acid regions of the cell would be reduced. Therefore, the cell would sustain a high negative potential until the internal pH increased to a level which would result in a slowing down of the H^+ extrusion pump. The external solution when buffered at high pH would have an effect similar to unbuffered high pH solution, but with a greater capacity to reduce the passive H^+ influx in the acid regions of the cell. The hyperpolarization would, therefore, be greater with buffers.

There is evidence that HCO_3^- stimulates the activity of ATPase localized in the plasmalemma of the oxyntic cells of Necturus gastric mucosa (Wiebelhars et al., 1972) and the pancreatic tissue of the dog (Simon et al., 1972).

Simon et al. have also indicated that a variety of bases can stimulate ATPase, as a function of pK of the bases. However, there is no evidence as yet to indicate that HCO_3^- stimulates the activity of ATPase in the Nitella plasmalemma.

4. CO_2

A preliminary experiment (C.E. Barr, unpublished) showed that under bright light the membrane potential of Nitella was hyperpolarized considerably when the external solution was switched from a solution containing 1.0 mM HCO_3^- at pH 8.3 to the same solution plus 1.5 mM CO_2, pH 6.2. Usually a depolarization of 20 mV would be expected when the pH of the solution was changed from 8 to 6 (Kitasto, 1968). Therefore Barr's experiment suggests CO_2 alone caused the hyperpolarization and this effect more than overcomes the effect of pH change. When the external pH is very high, e.g. in Barr's experiment, the internal pH would also stay high due to the small H^+ influx. Under this condition, although a high membrane potential would be sustained, the internal pH very likely does not favor a high rate of H^+ extrusion. If the light intensity is also very high, the active photosynthetic process would even further increase the internal pH. The resulting internal pH would then definitely be too high for the active operation of the H^+ extrusion pump. The addition of CO_2 in this circumstance would bring the external pH

and also the internal pH down to a level which is more favorable for the active operation of the H^+ extrusion pump, and a hyperpolarization would be observed. In Chara, a hyperpolarization was observed when removing CO_2 from the solution at pH 5.7 (Findlay et al., 1969). This further suggests that the effects of CO_2 on the resting membrane potential may be primarily due to its effect on the internal pH. It is quite certain that CO_2 passes through the membrane quite easily since it is a small, neutral molecule; this has been confirmed by the high rates of photosynthetic CO_2 fixation of characean species (Smith, 1967, 1968).

II. OBJECTIVES AND EXPERIMENTAL STRATEGIES:

The present research strategy, at least to some extent, reflects the premise that H^+ extrusion is of primary importance in controlling the internal pH and only secondarily is concerned with the precise level of the resting membrane potential. Therefore any factors which lead to a change in the internal pH could be expected to alter the rate of H^+ extrusion and this in turn might lead to a change in the level of the resting membrane potential. The H^+ extrusion pump itself may be quite sensitive to internal pH, but in addition, there may be other factors which determine the activity of the pump. Any experiments to be done are mainly exploratory; if pronounced, reproducible effects on the resting membrane potential can

be induced by a given change in the environmental conditions, such effects will be analyzed to see if they are consistant with the above hypothesis. As indicated above the level of the resting potential will also reflect the rate at which H^+ passively enters the cell. Both these factors will be considered in interpreting the results.

A change of the internal pH in response to the change in external conditions may take time to occur. Therefore the change should be studied over relatively long periods, i.e. one hour or more. The difficulties of devising adequate strategies in terms of changes in the environment alone are apparent. That is why an attempt at injecting known materials into Nitella cells was also planned.

If internal pH is not the only factor involved in the regulation of the H^+ extrusion pump, the situation will be too complex to analyze. If the energy supply is a critical factor, a test of one of the hypotheses regarding the driving of the H^+ pump is possible: to examine the effect of far red light (710-715nm) on the membrane potential. Far red light is known to energize photophosphorylation but not photosynthetic carbon assimilation. A hyperpolarization by far red light would suggest a dependence of the pump on ATP, perhaps through an ATPase reaction.

Experimental Strategies

A. Changes in the external environment

(See Table 2 for the compositions of solutions indicated)

1. Changes expected to result in an increase in internal pH:

a. external pH changes with and without bicarbonate in weak light (45 foot - candles)

K pH 5.7 ---(a)---> K pH 7.2 ---(b)---> Low Carbon pH 7.2 ($0.2mM\ HCO_3^-$ added)

As proposed above, a hyperpolarization would initally be expected in response to the change in step (a), due to an increase in the external pH and the decreased passive H^+ influx. With time the internal pH should rise and the membrane potential should become more positive as the H^+ extrusion pump slows down.

In short term experiments, the internal pH would be further increased in response to step (b) due to the buffering effect of HCO_3^- at pH 7.2. If a hyperpolarization occurs, it could be due to an effect similar to that of (a) but with better pH control at the surface of the membrane. If a depolarization occurs, it might be possibly due to a large increase in the internal pH, and this pH is too high for the H^+ extrusion pump to operate actively, while other cations (predominantly K^+) continue to come into the cell at an appreciable rate.

b. increase of light intensity

45 foot-candles ----------> 200 foot-candles

If a hyperpolarization occurs, it is presumed to be

due to an increase in the internal pH brought about by removing CO_2 from the cytoplasm (and additional H^+ uptake by chloroplasts) with a more favorable pH for the H^+ extrusion resulting. The energy supply for active H^+ extrusion should also be considered here. If a depolarization occurs, it could be due to the increase in the internal pH to such a high level that H^+ extrusion becomes greatly reduced. If no change occurs, it would suggest that the 45 foot-candle is of sufficient intensity to saturate the processes involved. In this case dark-light transitions could be tried. Monochromatic light is to be used as the light source to confirm that the observed effects are related to photosynthesis.

2. changes expected to result in a decrease in the internal pH:

a. addition of carbon dioxide + bicarbonate in weak light (45 foot-candles) at pH 5.7

K pH 5.7 ---------- High Carbon pH 5.7
(0.2 mM HCO_3^- + 1.0 mM CO_2)

If cells are unable to fix carbon as fast as CO_2 enters the internal pH could decrease. If a depolarization occurs it could be attributed to the lowering of the internal pH and some interference with normal metabolism under these low external pH conditions. If no substantial

change occurs, the simple conclusion is that the large CO_2 supply has little effect on the electrogenic mechanism. This conclusion should be tentative since an enhanced extrusion may be balanced by an increased passive H^+ influx with this buffered solution.

b. external pH change with CO_2 in both weak (45 foot-candles) and strong light (200 foot-candles)

CO_2-testing solution pH 7.6 ---> CO_2-testing solution + CO_2 pH 6.8
(0.075mM CO_2 + 1.2mM HCO_3^-) (0.5mM CO_2 + 1.2mM HCO_3^-)

This pH range was chosen because no change in the resting membrane potential is expected in response to the pH change alone in the range of 7.6 to 6.8 (Kitasato, 1968). If a hyperpolarization occurs, it could be attributed to a lowering of the internal pH by CO_2 and resulting in a favorable pH for the H^+ extrusion pump to operate. If a depolarization occurs, it would be due to the lowering of the internal pH to a level below that for maximal operation of the extrusion pump.

Different responses may occur in weak light and strong light since the internal pH is expected to be higher under the strong light than it is under weak light.

c. external pH change with CO_2 at low K^+ concentration

in weak light (45 foot-candles) and strong light (200 foot- candles)

2 Na solution pH 8.3 ----> 2 Na solution + CO_2 pH 6.5
(1.0mM HCO_3^-) (0.8mM CO_2 + 1.0mM HCO_3^-)

The K^+ fluxes in the previous solution (CO_2 testing solution) may be quite high and, therefore, hide effects due to the H^+ fluxes above. The 2 Na solution contains only 0.1mM K^+.

B. Injection of material into the cells

By injecting substances directly into the cells, their effects on the membrane potential through some internal action could be detected. However, since the materials could only be injected into the vacuole in the present study, their movement into the protoplasm is uncertain. This is because of the lack of the knowledge about the tonoplast's permeability to the materials to be injected. In Nitella the pH of vacuole is about 5.5 (Hirakawa and Yoshimura, 1964) and the protoplasmic pH is at least 6.3 (Rent et al., 1972). Therefore, the acidic material when injected into the vacuole might not cause a lowering of the protoplasmic pH.

Glyceraldehyde 3-phosphoric acid which was suggested by Spear et al. (1969) as a possible energy source for H^+ extrusion pump was injected, but, as indicated above, there is no certainty that it can readily enter the protoplasm.

Dihydroxyacetone, which is a sugar, was also injected in the hope that it may pass more easily into the protoplasm and be phosphorylated there; dihydroxyacetone phosphate can in turn be converted to its isomer, Glyceraldehyde-3-phosphate.

3-phosphoglyceric acid which is the product in the mechanism proposed by Spear et al. (1969) was also injected to see if it might depolarize the membrane since it is a product of the reaction proposed above.

In various animal cells, cyclic AMP has been reported to have effects on the permeability of the membrane to ions as well as small molecules (reviewed by Jost and Rickenberg, 1971). Although there is no indication that cyclic AMP is related to the H^+ extrusion pump, the injection of this substance was also attempted.

MATERIALS: Culture of Nitella and selection of cells

Plants of Nitella clavata Kutz were cultured in a solution which has the composition shown in Table 1. The cultures were kept in one-gallon jars under 16 hours of illumination of about 120 foot-candles, measured at the solution surface, alternated with 8 hours of darkness. Light was provided by equal numbers of Sylvania Gro-lux and cool white fluorescent lamps. The cultures were aerated, and small snails were present. The internodal cells, usually from the second to the fourth internode below the growing tips, were cut free from the neighboring internodal and branch cells. The cells were

then conditioned in K pH 5.7 solution or sometimes in other solutions (table 2), in culture dishes and kept at 22°C under 45 foot-candles cool white fluorescent illumination. If required by some specific experiments, the cells were then transfered to other solutions, also under 45 foot-candles cool white fluorescent light at 22°C.

The postharvest age of the cells used for experiments varied from 2 to 16 days. Only the cells showing active cytoplasmic streaming at room temperature were used. The size of the cells ranged from 1.7 to 3.0cm in length and 580 to 800μm in diameter.

METHODS

Unless otherwise indicated, all the experiments were carried out under 45 foot-candles cool white fluorescent light at room temperature (about 27°C).

Resting Membrane Potential

The resting membrane potentials of the cells were measured with Ag-AgCl microelectrodes which were connected to a General Radio Company 1230 A electrometer. The electrometer was in turn connected to an Esterline Angus A 6016 strip chart recorder.

By means of a Narashige PN-3 glass microelectrode puller, the measuring electrodes were made from the glass microcapillary of about 1mM outside diameter; the tips were drawn to about

15μm. The reference electrode was an Ag/AgCl electrode inserted into a wick type Coleman reservoir, (ordinarily used as part of a reference electrode for a Coleman pH meter). Both the measuring electrode and the reference electrode were filled with artificial cell sap (80mM KCl; 30mM Nacl 5mM $CaCl_2$). Silicone grease was used to seal the opening of the electrode into which the silver wire was inserted, to prevent evaporation of the filling solution.

For experimentation, a plexiglass trough was used to hold the cell. The trough has a groove of 0.7cm in width and 7cm in length. A continous flow of experimental solution was delivered into the trough from a reservoir through a polyethylene tubing. The flow rate (ca. 2 ml/min) was regulated with an adjustable tubing clamp so that the cell was always immersed in the solution. The flow of the solution was facilitated through use of a strip of filter paper placed at the end of the trough; this acted as a siphon.

The difference in electrical potential between the measuring microelectrode and the reference electrode when each was in a solution corresponding to the experimental situation was measured in the following way (this is equivalent to the "blank" in a chemical assay): The reference electrode was placed downstream in the trough to eliminate the effect of the possible leakage of solution from the tip of the reference electrode. The tip of the measuring electrode was stuck into a shallow polyethylene cup filled with artificial cell sap;

the cup was placed on the trough. A strip of filter paper was used as a salt bridge to electrically connect the cup to the solution in the trough in order to complete the circuit between the two electrodes. During the measurement solution was allowed to flow through the trough in the usual way; in this way any artificial cell sap siphoning into the trough was removed. Only the electrodes which had potentials between $^{+}$16mV and $^{-}$16mV were used. These measurements were made at the beginning and at the end of each experiment.

The cell, after being checked for protoplasmic streaming, was put in the trough. With the aid of a dissecting microscope (Titan 1218), a micromanipulator was used to insert the measuring electrode into the vacuole of the cell. The reference electrode was located near the end of the trough. The potential measured is actually the potential difference between the vacuole and the external solution, even though it is commonly refered to as the membrane potential. When pH measurements of the flowing solution became necessary, a small pH electrode and a reference electrode were placed upstream (relative to the cell) in the trough. After the membrane potential had reached a steady level, the experimental procedure was then started.

Illumination by Monochromatic Light

Except for illumination, the conditions used here were the same as those described under "Resting Membrane Potential". A Bausch and Lomb high intensity grating monochromator (33-86-25-02) with a Xenon arc lamp (33-86-20-01) was used to

generate the monochromatic light. The width of the slits were set so that the illumination at all wavelengths used provided the same quantal flux density, viz., 1.5×10^{-9} Einstein/cm^2 sec.. The energy fluxes of each wavelength were calibrated with a thermopile-type radiometer (Yellow Springs Instruments). A Corning glass filter (3-74) was used to eliminate 2nd order wavelengths for wavelengths above 650 nm, and a thin glass plate (ca. 2 mm) was used at shorter wavelengths. The use of the latter eliminated most of the radiation below 350 nm and could provide a greater light intensity than when the Corning filter was used. During the experimentation and energy flux measurements, the emitting end of the light elbow on the monochromator was situated 5 cm above the cell or the sensor.

The wavelengths used and the radiation flux densities of each are shown in Table 3. Even without any disturbance, the energy flux varies with time by itself. The probable error involved in each energy flux measurement is estimated to be $\pm 10\%$.

Injection of Solution into Nitella Cells

While the resting potential was being measured a microcapillary tube (similar to the one serving as the salt bridge of the measuring electrode) was used to inject small amounts of solutions into the cell.

The injecting microcapillary was made in the following way;

1) One end of a glass microcapillary of 1.0 mm O.D. was drawn to a tip of about 10 µm while the shank of the microcapillary tube was slightly narrowed in order to fit fairly tightly over a stainless steel tube (0.49 mm O.D.) inserted inside the glass capillary. The other end of the metal tube extended 2 or 3 cm outside the glass and could be used for attachment to polyethylene tubing.

2) Epoxy or Zipbond contact cement (Tescom Co.) was used to seal the metal tubing and the glass together. The neck prevented excessive contact between the cement and the solution to be injected into the cell.

3) After the seal had been dried, the free end of the metal tubing was inserted into polyethylene tubing (O.D. 0.43 mm); The other end of the polyethylene tubing was pushed over the end of the hypodermic needle of a high pressure 25 µl syringe (Precision Sampling, series C, pressure-lok). The syringe was driven by a Sage syringe pump (model 341). The whole system, from the tip of the microcapillary to the syringe was then filled with the solution to be injected.

The rate of injection of solution into the vacuole was regulated by the flow rate of the pump. The flow rate, when calibrated for the Precision Sampling 25 µl syringe, ranged from 0.2 µl per minute to 7.0 µl per hour. Phenol red was injected into the vacuole of one cell to check the operation of the whole injection system.

CO_2 Fixation

One-day old cells were put in finger bowls containing experimental solution, with 4 cells in each of 4 finger bowls. The K_c solution contained 0.1 mM HCO_3^- (4.5 Ci/Mole) and was bubbled with CO_2 in a radioisotope hood until the pH was lowered to 5.45. The finger bowls were sealed by nesting a second bowl on top and sealing with silicone grease. They were then kept at 22° C under 45 foot-candles cool white fluoreseent light for 17.5 hours. When the experimental period ended, the pH of the solution in the finger bowls was measured immediately after taking the cells out of the solution.

The cells were then rinsed 4 times, 15 minuted each, in the unlabled K_c solution. This procedure could thus remove any external radioactivity. Each cell was then torn apart in 0.5 ml of the "killing" solution (10 ml H_2O, 10 ml 95% ethyl alcohol, 60 ml 1.0 M $NaHCO_3$) in a planchet. The planchets were kept in the hood for 16.5 hours to evaporate.

The standard solution was prepared by adding 1 ml of 10 mM $NaH^{14}CO_3$ (4.5 Ci/Mole) to 99 ml of the "killing" solution. A volume of 0.5 ml of the standard solution was delivered into a plachet and treated the same way as was the cellular material in order to provide the same geometry and self absorption for the radioassay. Both the cellular material and the standards were counted under a GM tube with a Nuclear-Chicago 8703 decade scaler.

When analyzing the results, the dilution effects of nonradioactive CO_2 which stayed in the gaseous phase while CO_2 was being bubbled into the experimental solution, as well as the CO_2 in the aqueous phase were taken into account.

TABLE 1. COMPOSITION OF *NITELLA* CULTURE SOLUTION.

3.0 mM $CaCl_2$

1.0 mM $MgCl_2$

0.2 mM KCl

2.0 mM $NaHCO_3$

0.2 mM KNO_3

0.004 mM NaH_2PO_4

4.0 mM tris - (hydroxymethyl) - aminoethane neutralized with HCl to pH 7.0

1 ml/liter micronutrient stock solution

Micronutrient stock solution: filtrate from 50 g Brockport brown soil boiled 30 min. in 1.0 liter 6.0 mM ethylene-diamine tetraacetate at pH 8.0.

TABLE 2. COMPOSITION OF SOLUTIONS.

Concentrations are millimoles per liter. The K pH 7.2 solution was neutralized with KOH to pH 7.2 and used immediately.

Ion or Molecular	K	K	Low Carbon	High Carbon	CO_2-testing	CO_2-testing + CO_2	2Na	2Na + CO_2	K_c	D-4
pH	5.7	7.2	7.2	5.7	7.6	6.8	8.3	6.5	5.45	5.7
CO_2	-	-	0.032	1.0	0.075	0.5	0.013	0.8	0.9	-
HCO_3^-	-	0.03	.2	0.2	1.2	1.2	1.0	1.0	0.1	-
Cl^-	1.5	1.5	1.3	1.3	1.3	1.3	3	3	1.5	1.5
SO_4^{-2}	-	-	-	-	-	-	1.05	1.05	-	-
$H_2PO_4^-$	-	-	-	-	-	-	-	-	-	0.5
Na^+	0.1	0.1	0.1	0.1	1.1	1.1	2	2	0.1	0.135
K^+	1.0	1.0	1.0	1.0	1.0	1.0	0.1	0.1	1.0	1.35
Mg^{+2}	0.1	0.1	0.1	0.1	0.1	0.1	0.1	1.0	0.1	0.135
Ca^{+2}	0.1	0.1	0.1	0.1	0.1	0.1	1.0	1.0	0.1	0.135

TABLE 3. ENERGY FLUX DENSITY OF MONOCHROMATIC LIGHT.

The desired energy flux densities at all the wavelengths give the quantal flux density of 1.5×10^{-9} Einsteins/cm^2sec.

λ (nm)	440	475	525	575	625	675	710	715
Energy flux density desired (Ergs/cm^2sec)	4000	3680	3340	3060	2810	2600	2470	2450
Energy flux density measured (Ergs/cm^2sec)	3900 ±400	3750 ±400	3300 ±300	3100 ±300	2800 ±300	2600 ±300	2500 ±300	2450 ±300
Quantal flux density calibrated (Einsteins/cm^2sec x 10^{-9})	1.47 ±0.15	1.53 ±0.15	1.48 ±0.15	1.52 ±0.15	1.50 ±0.15	1.50 ±0.15	1.52 ±0.15	1.50 ±0.15

RESULTS AND DISCUSSION

A. Changes in the external environment

1. Changes expected to result in an increase in the internal pH

a. External pH changes with and without bicarbonate in weak light (45 foot-candles):

Table 4 is compiled from the experiments in which the cells were subjected to an increase in the external pH from 5.7 to 7.2 and subsequently to the HCO_3^- buffer at pH 7.2; the solution sequence was reversed in returning to pH 5.7. A period of 2 hours was allowed for each solution.

The membrane potential (E_m) was hyperpolarized by about 15 mV when the external pH was changed from 5.7 to 7.2. This result is in agreement with that obtained by Kitasato (1968). This hyperpolarization is attributed to the decreased H^+ passive influx in high pH solution. The internal pH would also be increased as a result.

When the cells were subjected to the HCO_3^- buffer, E_m was further hyperpolarized by about 20 mV. It may be tentatively concluded here that the bicarbonate acts as a sink for extruded H^+ which otherwise would reenter the cell. The suggested increase in internal pH in this case may be relatively small and this should result in only a

slight decrease in H^+ extrusion. However, there should be a greater reduction in the return current of H^+ into the cell because of the removal of extruded H^+ by bicarbonate. The observed hyperpolarization is consistent with this explanation.

Table 4 also shows that the responses of E_m to the external pH changes were reversible. Usually there was no lag period in the response of E_m to the removal of HCO_3^- from the external solution. This suggests that the effect of the HCO_3^- buffer is not directly concerned with the carbon metabolism but is due to external buffering by bicarbonate.

b. Increase of light intensity:

In order to examine the influence of light intensity on the membrane potential and its possible effect on the internal pH, cells were preconditioned under 45 foot-candles at three different pH levels and then subjected to the higher light intensity at the same pH.

Table 5 shows results obtained under these conditions. The increase in light intensity caused large depolarizations at pH 8.3 and pH 7.2 of about 30 and 35 mV, respectively. It should be noted that the 2Na solution is a low K^+ solution while the Low Carbon solution is a high K solution. However, at pH 5.7 the E_m did not respond to the increase

in light intensity in both the presence and absence of an external carbon supply.

As related to the hypothesis that internal pH is an important factor in how fast the H^+ pump operates, the cells conditioned at pH 7.2 and 8.3 would have rather high internal pH values due to the decrease of inward H^+ passive flux expected when the external pH is high. The increase in light intensity under these conditions would further raise the internal pH due to the removal of CO_2 and the additional H^+ uptake by the chloroplasts. The operation of the H^+ extrusion pump would slow down appreciably, and a depolarization would occur.

At pH 5.7 the simplest explanation for the insensitivity of the membrane potential to the increased light when an abundant supply of carbon dioxide is available is that E_m changes do not depend on the photosynthetically produced carbon compounds. Carbon fixation rates in characean species are quite high. Smith (1967a), in his study of the photosynthetic CO_2 fixation by Nitella translucens found that with 0.2 mM CO_2 + 0.3 mM HCO_3^- present at pH 6.3 maximal photosynthesis occured at 13,000 ergs/cm^2 sec of fluorescent light. Raven (1969) also found that when 1.0 mM CO_2 was present in the external solution at pH 6 photosynthetic carbon fixation was light-saturated at 10,000 ergs/cm^2 sec of 440 nm light. Therefore in the present study the light intensity of 45 foot-candles

(measured as 380 ergs/cm^2 sec on the YSI radiometer) presumably should not have saturated photosynthesis in High Carbon pH 5.7 solution. However, in the CO_2 fixation experiment (see section C below), which was carried out in a solution similar to the High Carbon solution, the rate of carbon uptake in 45-foot-candles cool white light was quite high, about 30 p mole/cm^2 sec. This would indicate that light at 45 foot-candles was not a limiting factor in carbon fixation. However, it is possible that carbon was fixed under these conditions but not reduced to the carbohydrate level. Oxygen evolution measurements are needed to confirm this.

The insensitivity of E_m to the increase in light intensity when the cells were conditioned at pH 5.7 perhaps is an indication of a high buffering capacity of the cytoplasm under this condition.

An attempt to link the effect of light intensity to photosynthesis was made by studying the E_m level under red (675 nm) and far red (715 nm) light. In red light noncyclic photosynthetic electron transport is possible; therefore both ATP formation and CO_2 fixation can take place. In far red light only cyclic electron transport is possible; ATP formation, but not CO_2 fixation, can occur.

Using the 2Na pH 8.3 solution the results obtained under red and far red light of same quantal flux density are shown in Table 6. Under these conditions Nitella cells

absorbed 91% of the 675 nm light and 52% of the 710 nm light. It is apparent that for a substantial depolarization to occur the noncyclic electron flow is necessary.

To further examin the question of whether the depolarizing effect of light on the membrane potential is linked to photosynthesis, an action spectrum was obtained in 2Na solution. Fig. 1 shows the depolarizing effects of various wavelengths of the same quantal flux density on one cell. The action spectrum of the depolarizing effect on the membrane potential appears to be consistent with photosynthesis, but the relatively small differences among the different wavelengths suggests that the light intensity was near saturation. Both blue and red light best promoted depolarization. The slight depolarization of the membrane potential for 710 nm is perhaps due to the overlapping of the absorption bands corresponding to the noncyclic (675 nm) and the cyclic (710 nm) components of photosynthesis (cf. Table 6). The precise level of E_m at each wavelength is shown in Table 7.

Fig. 2 shows the relation of the intensity of 675 nm light to the degree of depolarization of the membrane potential. It appears that the full intensity (2600 Ergs/cm^2 sec), which was also used in the study of action spectrum, was near saturation for the depolarization. This is consistent with the rather flat action spectrum, Fig. 1.

2. Changes expected to result in a decrease in the internal pH.

a. External Addition of CO_2 + HCO_3^- in weak light (45 foot-candles):

Table 8 shows the E_m values when the cells were bathed in K pH 5.7 solution and the changes which occured upon introducing the High Carbon pH 5.7 solution and allowing the membrane potential to stabilize. On the average a slight depolarization was observed but this depolarization was not significant.

The High Carbon pH 5.7 solution, which is a CO_2 + HCO_3^- buffer, is expected to provide an ample supply of H^+ and thus increase the inward passive H^+ flux. It also would provide CO_2 which will enter the cell rapidly. The membrane potential would therefore be depolarized to a level at which the passive H^+ influx becomes balanced by the H^+ extrusion pump. On the other hand, if a very capable H^+ extrusion pump is present in the cell, the lowering of the internal pH should promote the activity of the H^+ extrusion pump and hyperpolarize the membrane potential. The fact that in all the experiments a transient hyperpolarization was always observed agrees with this reasoning; that is, the lowering of the internal pH would initially promote the H^+ extrusion pump but in time a self-adjustment occurs and the E_m stabilizes at a slightly

depolarized level. However, the changes in E_m are relatively small on the average. In Exp. 28b, periodic small hyperpolarizations and depolarizations occurred while the cell was in High Carbon solution, but not in K solution. This oscillating behavior suggests some type of self-regulating mechanism.

While the cells were in High Carbon solution, action potentials sometimes occurred in between the initial hyperpolarization and the final steady state of the E_m. However, the removal of $CO_2 + HCO_3^-$ from the external solution, when changing back to K pH 5.7 solution, causes action potentials more frequently. This effect was not observed on the removal of HCO_3^- from the external solution at pH 7.2.

The fact that E_m is relatively insensitive to the lowering of the external pH with $CO_2 + HCO_3^-$ at 5.7 should also be considered in relation to the insensitivity of the E_m to the increase in light intensity at this pH. Perhaps this can be attributed to the greater buffering capacity of the cytoplasm when the cell is conditioned in pH 5.7 solution. This will be discussed in more detail under Conclusions.

b. CO_2 addition at neutral pH in both weak and strong light:

Table 9 shows the responses of E_m when CO_2 was added

to an approximately neutral solution, buffered with bicarbonate so that only a small pH change occured, i.e., from 7.6 to 6.8. The pH range was chosen because no change in E_m is expected in response to the external pH change alone in the range of 7.6 to 6.8 (Kitasato, 1968). In weak light a depolarization was observed in response to the CO_2 addition. On the other hand, in strong light a slight hyperpolarization was the usual response to the addition of CO_2. The mean value of E_m in pH 6.8 solution includes one very low value and so the results are confusing. More experiments need to be done to clarify the results.

One tentative conclusion can be drawn here: that the effect is due to the entry of CO_2 into the cell; in weak light this might result in a lowering of the internal pH to a harmful level, too low for the operation of the H^+ extrusion pump. This might explain the depolarization. In strong light the initial internal pH was high in CO_2-testing pH 7.6 solution, the addition of CO_2 would bring about a slight decrease in the internal pH and thus promote the H^+ extrusion pump; here a hyperpolarization would be expected.

c. External pH changes with CO_2 at low K^+ concentration in weak light (45 foot-candles) and strong light (200 foot-candles):

Table 10 shows the E_m changes in response to the addition of CO_2 to the 2 Na solution, which contains only 0.1 mM K^+. The K^+ fluxes in the high K^+ (1.0 mM) solutions such as CO_2-testing solution, may be quite high and therefore tend to hide effects due to H^+ fluxes alone. Recently, Ryan and Barr (1973) have obtained evidence for an H^+/K^+ exchange pump.

Both hyperpolarizations and depolarizations were observed under weak light when CO_2 was added. In strong light, a hyperpolarization always occurred in response to the CO_2 addition to the external solution. However, this hyperpolarization was usually transient, and in two out of five cases, the final E_m value with CO_2 present was more positive than the original E_m , i.e., before CO_2 was added.

An attempt to link this E_m change to photosynthesis was made by comparing the E_m changes caused by CO_2 in red (675 nm) light, far red (715 nm) light and darkness. Table 11 shows the results obtained under these conditions. In red light, a steady hyperpolarized potential was established when CO_2 was added to the external solution; in both far red and darkness the final potentials were at a depolarized level. From this observation one might conclude that CO_2 was being fixed in red light with its products serving as the energy source for the H^+ extrusion pump; thus the membrane potential would be hyperpolarized.

However, this conclusion is probably not correct. It should be noted that before CO_2 was added the precise level of E_m was lowest in red light. If CO_2 is being converted to some organic carbon compound which is linked to the H^+ extrusion pump, the E_m in red light should be more negative than the E_m in both far red and darkness. However, this was not observed. As related to the hypothesis suggested that the internal pH is of primary importance in affecting the operation of H^+ extrusion pump, the internal pH in red light as well as in strong light must be higher than that in far red or darkness, due to the effectiveness of both red and strong light in removing CO_2 in the cytoplasm through CO_2 fixation. Moreover, if the external pH is very high as in the present study, the small passive H^+ influx would result in an even higher internal pH, which very possibly is too high for the active operation of the H^+ extrusion pump. The addition of CO_2 in this circumstance would therefore bring the internal pH down to a level which is more favorable for the active operation of the H^+ extrusion pump, and a hyperpolarization would be observed. In darkness and far red light the increased passive H^+ influx due to the lowered external pH without a commensurate change in H^+ extrusion probably explains the depolarization. These results are somewhat clearer than those obtained for the same solution in bright light (Table 10).

In considering the ambiguous responses of E_m to the CO_2 addition in weak light and the sometimes transient hyperpolarization observed in bright light, the possible involvement of K^+ in activating the H^+ extrusion pump is another factor that must be considered. This is discussed further under Conclusions.

B. Injection of materials into the cells

As shown in Table 12, there was no significant change in E_m observed in response to the injection of various substances into the vacuole.

Although the concentration of Glyceraldehyde-3-phosphoric acid was increased to as high as 22 mM, which is perhaps 30 times more concentrated than the normal level (cf. Bassham and Krause, 1969), there was no response in E_m. The vacuolar pH must have been lowered appreciably since the above substance was injected into the vacuole in the acid form at pH 2.0. The lack of effect on E_m suggests that acid-base control also occurs at the tonoplast as Raven and Smith (1973) have proposed.

C. CO_2 Fixation

The mean rate of CO_2 uptake and fixation by 12 one-day old cells in Kc solution at an average pH of 5.6 under 45 foot-

candle illumination was 32 ± 1 μ moles/cm^2 sec. During the 17.5-hour experimental period the cells consumed about one-fourth of the CO_2 present; the average H_2CO_3 concentration in the aqueous phase was about 0.5 mM during this period. This rate is equal to the highest CO_2 fixation rates obtained by Smith (1967a) for Nitella translucens under 13,000 ergs/cm^2 sec. It thus appears that young cells of Nitella clavata have high rates of photosynthesis even under relatively weak illumination. As mentioned above the YSI radiometer reading for the 45 foot-candle illumination was only 380 ergs/cm^2 sec. This may be somewhat in error, but it seems quite unlikely that the energy flux density is greater than 1,000 ergs/cm^2 sec.

TABLE 4. EFFECT OF A pH INCREASE FROM 5.7 TO 7.2 ON THE MEMBRANE POTENTIAL WITH AND WITHOUT BICARBONATE PRESENT AT pH 7.2.

For the sequence of solution changes read from left to right in the table. The K pH 7.2 solution was neutralized with KOH to pH 7.2 and used immediately in the experiment. Low Carbon pH 7.2 solution had 0.2 mM HCO_3^- added to it. See Table 2 for the complete compositions. Cells with atypical responses are shown on the bottom of the table. The "prime" designation appended to an Exp. No. means that it is the second trial on one cell. (Act. Pot. = Action Potential, Low C = Low Carbon).

					E_m, -mv					
Exp. No.	Cell Age, Days	K pH 5.7	Max. K pH 7.2	Act. Pot.	Final K pH 7.2	Max. Low C pH 7.2	Act. Pot.	Final Low C pH 7.2	Final K pH 7.2	Final K pH 5.7
26d'	2	151	179	-	177	207	-	189	183	149
26e	3	146	173	-	161	199	-	194	161	138
26f	4	155	181	+	165	185	-	175	165	155
26g	3	168	193	+	168	183	-	178	168	168
26j	9	142	169	+	147	168	-	163	142	119
26l	8	141	172	-	163	196	+	184	166	146
26m	8	168	194	-	178	208	-	208	185	172
26m'	9	171	199	-	194	212	-	212	207	172
MEAN ± SEM		155±4	183±4		169±5	195±5		188±6	172±7	152±7
Cells with Atypical Responses										
26d	1	147	179	+	108	174	-	174	159	149
26h	5	141	184	-	91	91	-	91	10	93
26i	7	148	181	-	98	98	-	93	98	93
26k	6	144	172	+	11	21	-	21	27	27

TABLE 5. EFFECT OF LIGHT INTENSITY ON THE MEMBRANE POTENTIAL (2Na, LOW CARBON, HIGH CARBON AND K SOLUTIONS)

The light intensity was changed from 45 ft-c (cool-white fluorescent light) to 200 ft-c (45 ft-c cool-white + 155 ft-c incandescent). The membrane potentials under the higher intensity light stabilized after about 50 minutes; these are the potentials listed. The cells in the High Carbon solution at pH 5.7 were conditioned in K pH 5.7 solution; the membrane potentials of the cells in the K solution under 45 ft-c before the start of the experiment are also listed. The other cells were conditioned in the same solutions as used in the experiments. For the sequence of light conditions used, read from left to right in the table. (Low C = Low Carbon, High C = High Carbon).

			E_m, -mv		
Exp. Soln.	Exp. No.	Cell Age, Days	45 ft-c	200 ft-c	45 ft-c
	31b	10	225	145	
	31f	4	155	138	152
2Na	32a	8	171	154	
pH 8.3	32b	10	162	127	
	32c	6	154	146	135
	32d	7	153	118	
	34a	4	160	122	158
	34b	14	148	133	
	MEAN ± SEM		166 ± 9	136 ± 4	
	29d	8	184	173	
	29f	5	173	118	
Low C	29h	2	203	164	
pH 7.2	46a	5	206	161	196
	46b	6	205	175	195
	46c	7	217	186	203
	MEAN ± SEM		198 ± 7	163 ± 10	
		K pH 5.7 45 ft-c			
High C	44a	4 (173)	193	197	183
pH 5.7	44b	2 (159)	174	174	172
	44c	5 (170)	163	158	173
	MEAN ± SEM		181	181	176
	45a	1	161	156	158
K	45b	6	157	152	147
pH 5.7	45c	7	166	179	167
	MEAN		161	161	157

TABLE 6. EFFECT OF 675 nm AND 715 nm MONOCHROMATIC LIGHT ON THE MEMBRANE POTENTIAL. (2Na pH 8.3 SOLUTION).

The superscript apended to the E_m value indicates the sequence of lighting conditions. In Exp. 31e E_m value in 45 ft-c cool-white was used as standard and was checked before each lighting condition and afterwards. The E_m value in 45 ft-c listed is the mean value obtained. In Exp. 42a and 42b, E_m value in dark was checked before and after each monochromatic wavelength, with the E_m value in 45 ft-c only being measured at the start of each experiment. The E_m value in dark shown in table is the mean obtained. In Exp. 31, 42a, and 42b the cells were also subjected to the CO_2 addition at 675 nm; 675 nm and 715 nm; dark, 675 nm and 715 nm, respectively; those results are shown in Table 11. The duration of each treatment was about 90 minutes. The intensity of the monochromatic light was (1.5 ± 0.15) Einsteins/cm^2 sec, which was equivalent to 2600 Ergs/cm^2 sec for 675 nm.

		E_m, -mv			
Exp. No.	Cell Age, Days	45 ft-c	Dark	675 nm	715 nm
31e	5	171	192^2	136^3	196^1
42a	7	185^1	170	153^2	170^3
42b	6	192^1	210	159^2	216^3

TABLE 7. THE DEPOLARIZING EFFECT OF VARIOUS WAVELENGTHS OF MONOCHROMATIC LIGHT AS COMPARED TO DARKNESS (2Na pH 8.3 SOLUTION).

These results were obtained in two experiments on the same Nitella internodal cells. E_m values in darkness were measured both before the monochromatic light sequence and afterwards. The dark value corresponding to a given monochromatic light value was obtained by linear interpolation of the two dark values, i.e. a linear change in the dark value with time was assumed. The radiant flux density was $(1.5 \pm 0.15) \times 10^{-9}$ Einsteins/cm^{2}sec; this corresponds to 3900 ± 400 ergs/cm^2 sec for 440 nm light and 2600 ± 300 ergs/cm^2 sec for 675 nm.

	DAY 10			DAY 12		
nm	Sequence	E_m, -mv	Depolarization, mv	Sequence	E_m, -mv	Depolarization, mv
DARK	before	199	0	before	190	0
440	2	145	49	2	129	63
475	6	132	53	4	123	66
525	4	142	48	3	149	45
575	5	141	48	6	138	54
625	1	147	47	5	130	59
675	3	144	49	1	126	65
675	8	123	58	8	131	53
710	7	169	14	7	166	20
DARK	after	177	0	after	176	0

TABLE 8. EFFECT ON THE MEMBRANE POTENTIAL OF ADDITION OF CO_2 + HCO_3^- TO K pH 5.7 SOLUTION AT THE SAME pH.

The High Carbon pH 5.7 solution contains 1.0 mM CO_2 and 0.2 mM HCO_3^- with the HCO_3^- replacing Cl^- in the K pH 5.7 solution. See Table 2 for complete compositions. The ΔE_m was obtained by subtracting the mean of the two values for the K solution from the value of the High Carbon solution. The "prime" designation appended to Experiment No. means that it is the second trial on the same cell, which was done after letting the cell rest overnight in K solution pH 5.7. For the sequence of solution changes read from left to right. (High C = High Carbon).

		E_m, -mv				
Exp. No.	Cell Age, Days	K pH 5.7	Max. High C pH 5.7	Final High C pH 5.7	Final, K pH 5.7	ΔE_m, mv
28a	13	158	164	150	165	+13
28a'	14	167	173	150	160	+14
28b	11	155	168	164	167	- 3
28c	4	149	164	136	159	+18
28d	6	165	174	169	163	- 5
28f	5	144	156	122	142	+21
28g	7	141	166	141	158	+ 9
28h	8	152	161	147	160	+ 9
MEAN ± SEM		154 ± 4	166 ± 2	147 ± 5	159 ± 3	9.4 ± 2.9

TABLE 9. THE EFFECT OF CO_2 ON THE MEMBRANE POTENTIAL AT NEUTRAL pH (CO_2-TESTING SOLUTION).

CO_2 was bubbled into the CO_2-testing solution at pH 7.6, containing 1.2 mM HCO_3^-. The lowering of the pH to 6.8 by the CO_2 addition is in itself assumed to have little or no effect on the membrane potential. See text and Table 2 for further details. ΔE_m was obtained by subtracting the mean of the two values for the pH 7.6 solution from the value at pH 6.8.

			E_m, -mv			
Exp. No.	Cell Age, Days	Light Intensity, ft-c	pH 7.6	pH6.8 (CO_2)	pH 7.6	ΔE_m
29a	7	45	191	182	193	+10
29b	10	45	188	165	175	+16
29d	8	200	180	209	173	-34
29e	10	"	218	221	226	+ 1
29f	5	"	161	101	174	+66
29g	6	"	196	207	192	-13
29h	2	"	189	196	184	-10
MEAN ± SEM for 200 ft-c			188±9	186±22	189±10	

TABLE 10. THE TRANSIENT HYPERPOLARIZING EFFECT OF CO_2 ON THE MEMBRANE POTENTIAL OF NITELLA CELLS IN LIGHT (2Na SOLUTION).

The 2Na solution contains 2 mM Na, 0.1 mM K and 1.0 mM HCO_3^-. The CO_2 is bubbled into the 2Na solution, lowering the pH from 8.3 to 6.5 and giving a H_2CO_3 concentration of 0.8 mM. The complete solution compositions are given in Table 2.

			E_m, -mv				
Exp. No.	Cell Age, Days	Light Intensity, ft-c	2Na pH 8.3	Max. 2Na + CO_2	Final 2Na + CO_2	2Na pH 8.3	
31a	6	45	211	183	183	died	
31d	16	45	142	187	167	162	
							E_m, -mv at 45 ft-c
31b	10	200	145	203	203	158	225
32a	8	"	154	201	107	149	171
32b	10	"	127	187	152	137	162
32c	6	"	146	179	161	147	154
32d	7	"	118	172	100	130	153
MEAN ± SEM at 200 ft-c			138 ± 7	188 ± 6	145 ± 19	144 ± 5	

TABLE 11. THE EFFECT OF CO_2 ON THE MEMBRANE POTENTIAL OF NITELLA CELLS IN MONOCHROMATIC RED AND FAR RED LIGHT (2Na SOLUTION).

The 2Na solution contains 2 mM Na, 0.1 mM K and 1.0 mM HCO_3^-. The CO_2 is bubbled into the 2Na solution, lowering the pH from 8.3 to 6.5 and giving a H_2CO_3 concentration of 0.8 mM. The radiation flux density is given in Table 3 and the solution compositions are given in Table 2. The potentials at pH 6.5 were the maximum and the steady state levels obtained, the latter at about 2 hours after the CO_2 addition.

			E_m, -mv			
Exp. No.	Cell Age, Days	Wavelength, nm	2Na pH 8.3	Maximum 2Na + CO_2 pH 6.5	Final 2Na + CO_2 pH 6.5	2Na pH 8.3
42a	7	675	150	182	177	155
42b	6	675	144	193	162	174
42a	7	715	170	195	162	170
42b	6	715	215	205	199	217
42b	6	DARK	217	211	187	212

TABLE 12. THE EFFECT OF INJECTING VARIOUS SUBSTANCES INTO THE VACUOLE OF NITELLA CELLS: CHANGES IN THE MEMBRANE POTENTIAL.

See Methods for details. The "prime" designation appended to an Experiment No. means that it is the second injection for that cell. (Inj. = Injection).

Exp. No.	Cell Age, Days	Substance Injected	Injection Rate, ml/hr	Duration, hr	Approx. Final Conc. in Cell, mM	Dark or Light	E_m, -mv Before Inj.	E_m, -mv At End Of Inj. Period	E_m, -mv After Inj. (hr)
7a	2	Distilled	3.8	0.5	...	L	160	200	
7b	5	H_2O pH 5.5	3.8	4.5	...	L	165	155	
5e	14	0.294 M	300	0.025	22.4	D	155		160 (1)
15a	2	DL Glycer-	2.5	2.3	17.1	D	130	135	
15a'	2	aldehyde-3- phosphoric acid pH 2.0	150 + 2.5	0.017 1.25	14.2	D	135	102	132 (1)
11a	9	"	0.2	3.5	0.7	L	63	63	
11a'	9	"	12	0.67	8	L	70	70	
15b	1	"	1.8	4.0	7.2	L	145	140	
6b'	2	0.0147 M	12	0.58	10.3	L	125	115	
6c	4	DL Glycer- aldehyde-3- phosphate, K salt pH 6.15	2.5	2.5	7.4	L	130	138	
9a	5	0.015 mM Di-	2.5	2.0	0.008	L	153	151	
9a'	6	hydroxy- acetone	150	0.033	0.008	L	154		154 (1)
8b	6	" (this cell conditioned in D-4 solution	3.5	2.8	0.02	L	103	103	
16a	7	0.0434 M 3-	2.5	0.5	5.4	L	140	95	110 (2)
16b	3	phosphogly-	"	0.9	10	L	123	123	
16c	9	ceric acid	"	1	11	L	95	100	
16d	6	pH 5.37	"	2	22	L	102	120	
16f	5		"	2	22	L	150	153	
19a	3	0.05 mM cyclic AMP in K soln. pH 4.5	2.5	2.5	0.03	L	128	120	

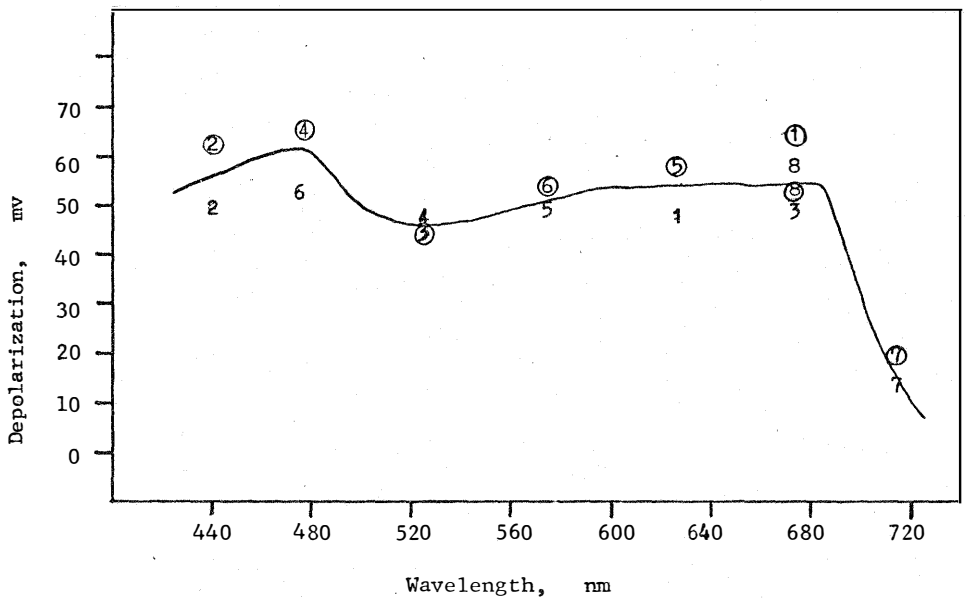


Fig. 1. Depolarizing effect of various wavelengths of monochromatic light on Nitella, as compared to darkness, in 2 Na pH 8.3 solution. The numbers on the curve indicate the sequence of the monochromatic wavelengths applied. The uncircled numbers indicate the values obtained from a 10-day old cell while the circled ones indicate those obtained from the same cell on day 12. Between days 10 and 12, the cell was kept under 45 foot-candles cool white fluorescent light. The precise values obtained are presented in Table 7.

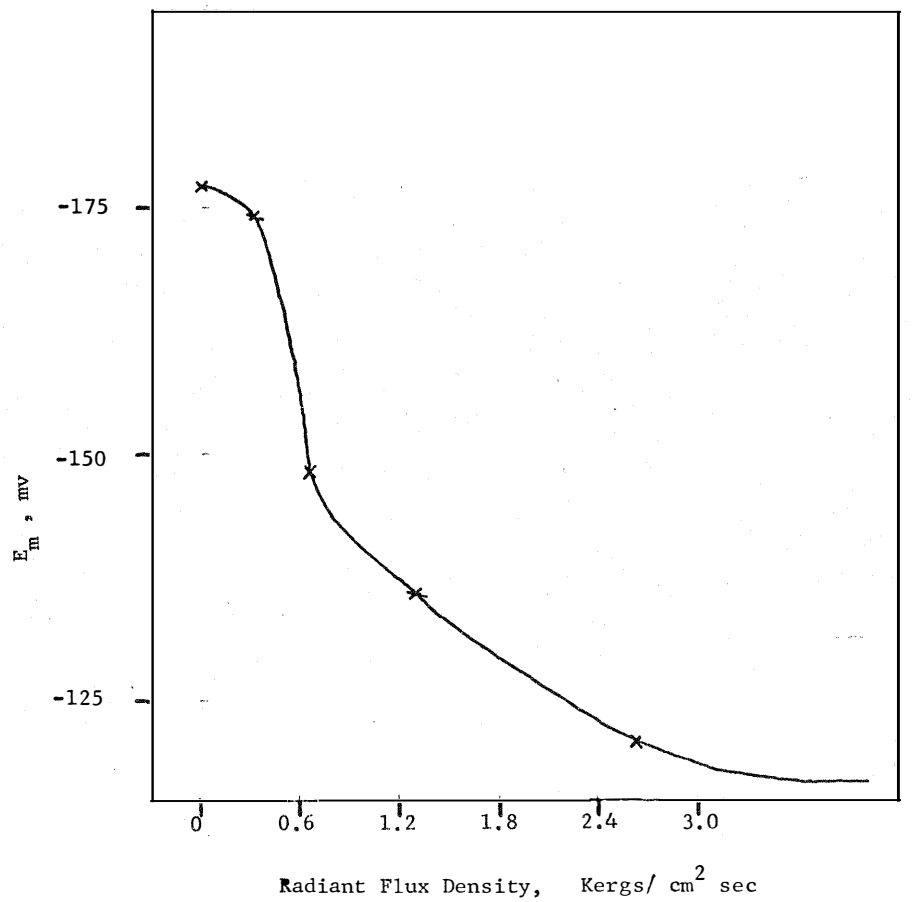


Fig. 2. The dependence of the level of the membrane potential on the intensity of 675 nm light. The results were obtained on a 10-day old Nitella cell in 2 Na pH 8.3 solution.

CONCLUSIONS

In the RESULTS and DISCUSSION section the experimental results were presented in their relation to a hypothesis: that the internal pH is regulated by the operation of the H^+ extrusion pump, which in turn would, in general, have a definite effect on the resting membrane potential. Some of the results are clearly interperable in terms of this hypothesis.

Since chloroplasts are known to take up H^+ when illuminated (Jagendorf and Uribe, 1969), it seems quite certain that increasing the light intensity would cause a depolarization. When the cells were conditioned at pH 7.2 or 8.3, the internal pH of these cells would already be high due to a small passive H^+ influx in high pH solutions. The increase in light intensity, which could increase photosynthetic rate and further raise the internal pH, would slow down the operation of the H^+ extrusion pump and thus result in a depolarization of E_m. At pH 8.3, the depolarization of 50 mV or more to a new steady level under fairly intense monochromatic light clearly supports the above reasoning. Even though the light at the various wavelengths appears to be almost saturating the dependence on photosynthesis as indicated by maxima in red and blue light is evident. On the other hand, when the cells were conditioned at pH 5.7 no significant effect of increasing light intensity or adding $CO_2 + HCO_3^-$ to the K solution was observed. In terms of the internal pH this would suggest a high cytoplasmic

buffering capacity under this condition. Support for this suggestion is available from other work recently completed. Ryan (M.S. Thesis, 1973) has found that the concentration of K^+ in Nitella cells conditioned in K pH 5.7 solution increased from 55 mM to 100 mM during the first two weeks after harvest. DiGregorio et al. (submitted to Plant Physiology) measured the Cl^- concentration under the same conditions and found the Cl^- concentration to be constant with age, at 85 mM. Since Cl^- is the only anion available in K pH 5.7 solution, the imbalance between K^+ and Cl^- concentrations found in Nitella cell must be attributed to the formation of organic anions in the cell. This represents indirect evidence for an increased buffering capacity, but there isn't any other explanation.

Another rather clear cut result is the hyperpolarizing effect of CO_2 on E_m at pH 8.3 which can be interpreted as a lowering of the internal pH and the subsequent stimulation of H^+ extrusion. However, its temporary nature is confusing and indicates that some other factors are involved. Recently, Ryan and Barr (1973) have reported that the H^+ extrusion pump is a H^+/K^+ exchange pump. Since the 2Na pH 8.3 solution contains only 0.1 mM K^+, the limited response of the E_m may mean that the K^+ concentration in 2Na solution is too low to sustain a high rate of H^+ extrusion very long. It is possible that the K^+ concentration near the outer surface of the cell membrane becomes depleted under these conditions. The fact

that, in strong light, the hyperpolarizing effect of CO_2 in the CO_2-testing solution, which contains 1.0 mM K^+, is quite stable, also supports the possible involvement of K^+ in the H^+ extrusion pump.

The results obtained in the present study do not favor the idea that photosynthetically-produced carbon compounds are rate-limiting for the operation of the H^+ extrusion pump. Photosynthesis is linked to the mechanism which maintains the resting membrane potential, but as the results have indicated, the coupling between these two processes appears to be manifested through changes in the internal pH. The fact that far red light (710-715 nm), which energizes photosynthetic ATP formation, did not hyperpolarize the membrane potential does not give support to the idea that an ATPase reaction drives the H^+ extrusion pump; it is of course possible that the ATP level is always adequate.

The hypothesis that H^+ extrusion pump is of primary importance in controlling the internal pH has obtained support from present study. However, more information about the precise change in internal pH brought about by an environmental change is necessary before a more confident conclusion is possible. Technical difficulties involved in measuring the cytoplasmic pH still exist and must be overcome if direct evidence is to be obtained. Perhaps more extreme environmental changes can be attempted, and if they do not cause damage or lethal effects, more definite internal changes would occur and lead to more striking changes in the membrane potential.

SUMMARY

In Nitella cells the H^+ extrusion pump is believed to be the mechanism for generating the electric potential across the plasmalemma. Raven and Smith (1973) have proposed that the primary role of the H^+ pump is to control the internal pH, with the level of the membrane potential (E_m) varying, depending on the H^+ pumping rate. The level of the resting potential would also depend upon the magnitude of the return current of H^+ into the cell; this in turn is dependent on the external pH.

The above hypothesis was tested by imposing external conditions which would lead to changes in internal pH and by observing the resulting changes in E_m. Some of the results were clearly consistent with the hypothesis: (a) Bright light depolarized the membrane as compared to darkness or weak light at pH 8.3; this is ascribed to an increase in the internal pH induced by photosynthesis and a consequent decrease in H^+ extrusion. (b) CO_2 addition under the above conditions caused a hyperpolarization even though the external pH was lowered from 8.3 to 6.5. (c) When cells were conditioned at pH 5.7 a large increase in organic anion content resulted, buffering the cytoplasm and, as predicted, this prevented any significant changes in E_m when bright light or CO_2 were introduced.

Studies with monochromatic light indicated that the action spectrum for membrane depolarization is similar to that for photosynthetically-induced changes of the cytoplasmic pH.

There is no evidence to indicate or suggest that the level of either ATP or carbon compounds is a rate-limiting factor in the operation of the H^+ pump under any of the conditions used in this study.

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